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# IFN-γ induces apoptosis in mouse embryonic stem cells, a putative mechanism of its embryotoxicity

Gang-Ming Zou,[1,2]* Marie-Françoise Reznikoff-Etievant,[1] François Hirsch[3] and Jacques Milliez[1]

[1]*Department of Gynecology/Obstetrics, Saint-Antoine Hospital, 184 rue du Faubourg St-Antoine, 75012,* [2]*Institut Jacques Monod, Department of Developmental Biology, 2 Place Jussieu, Tour 43, 75005 Paris and* [3]*CNRS ERS 1984, 19 rue Guy Môquet, 94801, Villejuif, France.*

It has been reported that interferon (IFN)-γ should inhibit *in vitro* mouse embryo growth by direct cell toxicity. However, the mechanism involved has not been clearly established. In the present study, this question was addressed using the embryonic stem (ES) cell model. It was found that IFN-γ induces a dose-dependent apoptosis in ES cells, as assessed by trypan-blue staining, by Annexin-V labeling and DNA analysis. Moreover, IFN-γ treatment cooperates with Fas-mediated apoptosis, a phenomenon that has been recently reported. As Bcl-2 oncoprotein functions as a death repressor molecule in an evolutionarily conserved cell death pathway, its expression was analyzed by flow cytometry. It was demonstrated that Bcl-2 is expressed in ES cells. When compared to untreated ES cells, IFN-γ-treated, apoptotic cells expressed a lower Bcl-2 level and a normal level of Fas, whereas surviving cells expressed a normal level of Bcl-2 but a lower Fas expression. Altogether, these data suggest that IFN-γ may influence early mouse embryo development by promoting apoptosis, which may constitute a novel mechanism of IFN-γ embryotoxicity.

**Key words:** apoptosis, bcl-2, embryonic stem cells, IFN-γ.

## Introduction

Apoptosis is as fundamental to cellular and tissue physiology as cell division and differentiation (Granville *et al.* 1998). It is an essential feature of normal physiology in a variety of organs and it plays an important role in fetal development (Lea *et al.* 1997). The well-defined loss of specific cells is crucial during embryonic development as a part of organogenesis (Gluksman 1951). There is also increasing evidence that regulated apoptosis is important during implantation and early pregnancy. Thus, an unresolved developmental question is how a cell knows whether it should survive or self-destruct. Determining the expression pattern of proteins that regulate cell survival is a critical aspect of understanding this process.

Interferon (IFN)-γ has been shown to be toxic to embryonic and trophoblast cells *in vitro* (Hill *et al.* 1987; Berkowitz *et al.* 1988; Haimovici *et al.* 1991), and it may play a role in reproductive dysfunction in unexplained recurrent abortion (URA) patients (Hill *et al.* 1995). Recently, IFN-γ was reported not to impair early embryo development, but to significantly inhibit blastocyst spreading (Cameo *et al.* 1999). γ-Interferon is able to induce apoptosis in various cells, such as vascular smooth muscle cells, normal endothelial cells and differential leukemic B-cell lines (Trubiani *et al.* 1994). IFN-γ inhibited HT29 human adenocarcinoma cell growth through Fas-mediated apoptosis, and STAT1 protein is required to up-regulate Fas and FasL expression during this procedure (Xu *et al.* 1998). γ-Interferon also activated Fas-mediated apoptosis in IL-6-dependent and IL-6-independent multiple myeloid cell lines through up-regulation of Fas antigen expression but did not alter the expression of Bcl-2 or Bax (Spets *et al.* 1998). However, there was also a report that IFN-γ inhibited apoptosis induced by wild-type p53, indicating that it could be an anti-apoptotic cytokine for myeloid cells in which apoptosis was induced by wild-type p53, cytotoxic anticancer agents or viability factor deprivation (Lotem & Sachs 1995). γ-Interferon in the culture suppressed human mast cell apoptosis and prolonged their survival in a dose-dependent manner (Yanagida *et al.* 1996). Thus these different results may demonstrate that the IFN-γ-mediated apoptosis is cell type dependent.

Bcl-2 was initially discovered as an overexpressed

*Author to whom all correspondence should be addressed at: Institut Jacques Monod, Laboratory no. 42, 2 Place Jussieu, Tour 43, 75005 Paris, France.

E-mail: zougm@francemultimedia.fr

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protein in human B-cell lymphoma arising as a result of a t(14;18) chromosomal translocation (Pegoraro *et al.* 1984; Tsujimoto *et al.* 1985). It is a 25 kDa integral membrane protein that has been localized to mitochondria, nuclear envelope/perinuclear membrane and the endoplasmic reticulum (Novack & Korsmeyer 1994). This oncogenic protein acts to inhibit apoptosis (Reed 1997). As Bcl-2 prevented programmed cell death induced by a wide array of signals, it may be that Bcl-2 is a central repressor of cell death with an important regulatory role during the development of many organs (Novack *et al.* 1994). Bcl-2 expression was detected in the embryo at day 8.5 (Novack & Korsmeyer 1994). In later stages of embryo development, Jurisicova *et al.* (1998) reported recently that the 1-cell-stage embryo (zygote) expresses Bcl-2; a small amount of Bcl-2 transcription may also occur during the 2-cell stage. In the blastocyst, Bcl-2 messenger RNA (mRNA) was predominantly expressed in the inner cell mass (ICM), however, a lower amount of Bad mRNA is expressed in the ICM. This result indicated that the expression of these two genes could play a role in controlling apoptosis within the ICM, thus providing for part of the regulatory capacity of the mammalian embryo. Prior studies of Bcl-2 in human fetal tissue found that its expression was more widespread in the embryo than in the adult, suggesting a role in morphogenesis (Lebrun *et al.* 1993). In ovary development, it is also proposed that Bcl-2 plays a role in ovarian cell functions such as cell proliferation and differentiation (Johnson *et al.* 1997). In the present study, using the embryonic stem (ES) cell model, we found Bcl-2 expression in the pre-implantation stage mouse embryo.

To understand the mechanism of embryotoxicity of IFN-γ, we use ES cells as a model in the present study. We found that the IFN-γ-mediated ES cell death occurred through apoptosis in a dose-dependent manner and that addition of antibody directed to Fas protein (present in ES cells; Zou *et al.* 2000) improved IFN-induced apoptosis. As the expression of Bcl-2 correlates with resistance to apoptosis, we also investigated the expression of Bcl-2, compared with Fas expression upon IFN-γ treatment.

## Materials and Methods

### *Cell and cell culture*

Mouse embryonic stem cell line CJ7 was derived from the blastocyst of the 129/SV mouse strain and adapted to grow in the presence of leukemia inhibitory factor (LIF; Nakayama *et al.* 1998). CK35 ES cells were derived from the 129/Sv strain by Dr Kress at the Pasteur Institute in Paris (Cohen-Tannoudji *et al.* 1998; Kress *et al.* 1998). The ES cells remain in an undifferentiated state in the presence of LIF in the culture medium. The cells were maintained on gelatinized tissue culture dishes (100 mm; Costar, Cambridge, MA, USA) in standard ES culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS; Gibco, Grand Island, NY, USA), 0.1 mmol L-glutamine, 150 μmol monothioglycerol (MTG), penicillin 100 U/mL, streptomycin 100 μg/mL, and leukemia inhibitory factor 1000 U/mL. The feeder cells were not used in our culture system and no ES cell differentiation or embryonic body (EB) formation was found in this maintaining culture system. The culture medium was changed every day, and the cells were passaged every 2 or 3 days.

### *Assay of cell death*

Cell death was assayed by a trypan blue exclusion method. In brief, $2 \times 10^5$ ES cells were placed in one well of a 12-well plate (Costar) and treated with 100, 500 or 1000 U/mL of mouse recombinant IFN-γ (R & D Systems Inc., Abingdon, UK). After a 24 h culture, the cells were washed with phosphate-buffered saline (PBS) and cell viability was examined by counting the live cell numbers. In the second series of experiments, $2 \times 10^5$ ES cells were cultured with 500 U/mL of IFN-γ, 2 μg/mL of Jo2, 500 U/mL of IFN-γ with 2 μg/mL of Jo2, or 2 μg/mL of Hamster immunoglobulin G (IgG) alone (control). After a 24 h culture, the cells were washed with PBS and the cell viability was examined by counting the live cell number.

### *Annexin V flow cytometry assay*

Annexin V was reported as a convenient probe for identifying apoptotic cells (Koopman *et al.* 1994; Wen *et al.* 1997). Embryonic stem cell ($1 \times 10^6$ cells/mL) medium was cultured in a 60 mm dish. In the negative control, only culture medium was added. In the test dish, IFN-γ at 500 U/mL was added. The cells were cultured in a 5% $CO_2$ incubator at 37°C, then collected at the time point of 24 h, washed twice with PBS-bovine serum albumin (BSA) and stained with Annexin V (fluorescein isothiocyanate (FITC) labeled). Annexin V binding was performed following the manufacturer's direction with a small modification in the procedure (Pharmingen, San Diego, CA, USA). Briefly, Annexin V (FITC labeled, 5 μL), propidium iodide (10 μL), and the binding buffer (400 μL) were added to a final volume of 500 μL, then analyzed by FACScan (Becton Dickinson, Sunnyvale, CA, USA) in 1 h.

### *DNA fragmentation assay*

To examine DNA fragmentation in the cells, CJ7 ES cells were cultured for 24 h in a medium (15%

FCS/DMEM) containing different concentrations of IFN-γ. Then the cells were collected and a DNA ladder kit (Genzyme; Cambridge, MA, USA) was used in the analysis. Briefly, $2 \times 10^5$ ES cells were cultured in a 12-well plate (Costar). After 24 h incubation with IFN-γ, the cells were washed with cold PBS and trypsinized with trypsin-ethylenediamine tetraacetic acid (EDTA; Gibco BRL, Gaithersburg, MD, USA). The genzyme TACS™ apoptotic DNA ladder kit was used to extract DNA from collected cells. The extracted DNA was dissolved in 50 µL Tris-EDTA (TE) buffer and electrophoresis was performed on a 1% agarose gel containing 0.05 µg/mL ethidium bromide.

### *Cell permeabilization and Bcl-2, Fas flow cytometry analysis*

Trypsinized ES cells were incubated with SB (1% w/v BSA (Sigma Chemical Co., St Louis, MO, USA) in PBS, pH 7.4) with saponin detergent (Sigma Chemical Co.) added to permeabilized cells. The ES cells were stained and washed in SB containing 0.03% saponin, and $1 \times 10^6$ ES cells were incubated with 1 µg hamster antimouse Bcl-2 monoclonal antibody (mAb; Pharmingen) in SB containing saponin, for 30 min at 4°C. They were then washed with PBS, incubated with FITC conjugate goat anti-Hamster IgG (Tebu Co.; Le Perray-en-Yvelines, France), and read on flow cytometry. To study the change in Bcl-2 level after IFN-γ administration, Bcl-2 was assessed in the cultured cell populations by gating the viable and non-viable populations as determined by forward and side scatter profiles (Tamaru *et al.* 1993; Pepper *et al.* 1998). For Fas flow cytometric analysis, $1 \times 10^6$ IFN-γ-treated ES cells were labeled with 1 µg of FITC-conjugated antimouse Fas mAb (Jo2 clone), incubated for 30 min at 4°C, washed with PBS, then evaluated using FACScan (Becton Dickinson, Sunnyvale, CA, USA). Actin was used as a control protein in flow cytometry assay.

### *Statistical analysis*

All data are presented as mean ± SD. The statistical analyses were performed using the Student's *t*-test.

## Results

### *IFN-γ affected ES cell growth*

We investigated the growth inhibition of IFN-γ on CJ7 and CK35 ES cells *in vitro*. As shown in Fig. 1, ES cell growth was inhibited by the addition of 500 or

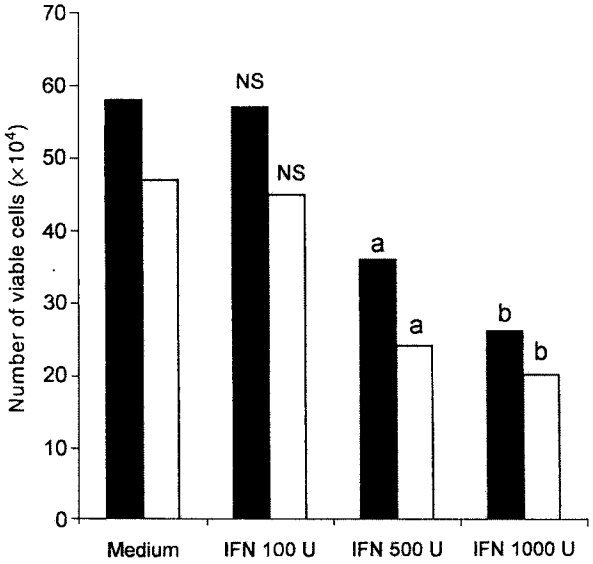


**Fig. 1.** Changes in the number of viable embryonic stem (ES) cells exposed to interferon (IFN)-γ. The ES cells were collected after 24 h culture in the absence (medium) or in the presence of 500 U/mL or 1000 U/mL of IFN-γ. Cell viability was determined by trypan blue exclusion assay. The results are the mean ± SD of three separate experiments. a, $P < 0.01$; b, $P < 0.001$; NS, not significant when compared to cells cultured in medium alone. (■), corresponds to CJ7 ES cell line; (□), corresponds to CK35 ES cell line.

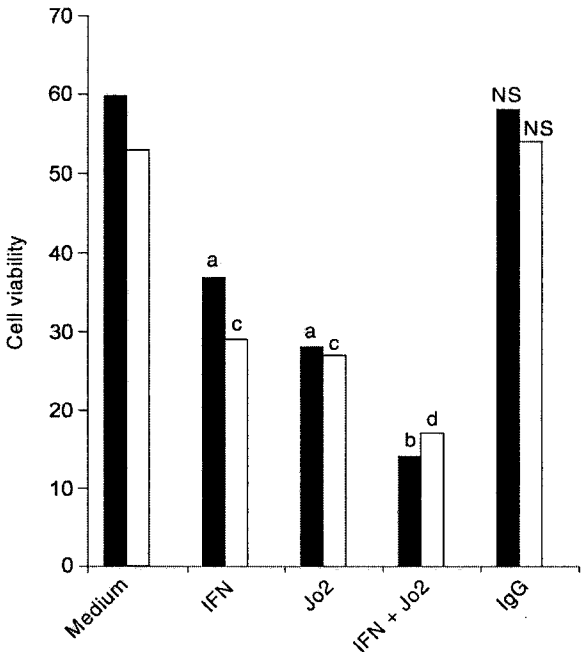


**Fig. 2.** Combining effects between interferon (IFN)-γ and Jo2 (anti-Fas monoclonal antibody (mAb)) in inducing embryonic stem (ES) cell death. Cells were cultured in the presence of hamster immunoglobulin G (IgG; 2 µg/mL), Jo2 (2 µg/mL), IFN-γ (500 U/mL), or a combination of IFN-γ (500 U/mL) and Jo2 (2 µg/mL). At 24 h, cells were harvested and the viable cell number was determined by trypan blue exclusion assay. The results are the mean ± SD of three separate experiments. a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.02$; d, $P < 0.05$; NS, not significant when compared to the medium. (■), Corresponds to the CJ7 ES cell line; (□), corresponds to the CK35 ES cell line.

1000 U/mL medium of IFN-γ. Live cell numbers were dramatically decreased when the dose of IFN-γ reached 500 U/mL as compared to control groups cultured without IFN-γ (in the CJ7 cell line: for 500 U/mL IFN-γ, $37 \pm 2.65 \times 10^4$ *vs* $56 \pm 3.21 \times 10^4$, $P < 0.01$, $n = 3$; for 1000 U IFN-γ, $28.33 \pm 1.53 \times 10^4$ *vs* $56 \pm 3.21 \times 10^4$, $P < 0.01$, $n = 3$; in CK35: for 500 U/mL IFN-γ, $26 \pm 5 \times 10^4$ *vs* $46.5 \pm 10.4 \times 10^4$, $P < 0.01$, $n = 3$; for 1000 U/mL IFN-γ, $20 \pm 8.5 \times 10^4$ *vs* $46.5 \pm 10.4 \times 10^4$, $P < 0.001$, $n = 3$). The IFN-γ at 100 U/mL had no obvious affect in inhibiting the growth of either strain of ES cells ($P > 0.05$ when compared with the control group). Interestingly, treatment of ES cells with anti-Fas mAb (Jo2; 2 μg/mL) increased the effect observed with IFN-γ (500 U/mL) in inducing ES cell death in the two different ES cell lines studied (Fig. 2). Under this condition, CJ7 ES cell viability dropped from $60.3 \pm 2.1 \times 10^4$ to $35 \pm 2.6 \times 10^4$ in the presence of 500 U/mL IFN-γ ($P < 0.001$, $n = 3$), to $28 \pm 2.6 \times 10^4$ in the presence of anti-Fas mAb ($P < 0.001$, $n = 3$). Similarly, CK35 ES cell viability dropped from $54 \pm 12.2 \times 10^4$ to $28.7 \pm 6.7 \times 10^4$ in the presence of 500 U/mL IFN-γ ($P < 0.02$, $n = 3$) and to $17.7 \pm 5 \times 10^5$ in the presence of 500 U/mL IFN-γ and anti-Fas mAb ($P < 0.05$, $n = 3$). It is of note that no significant differences were observed when comparing IFN-treated cells to cells treated with both IFN-γ and anti-Fas mAb.

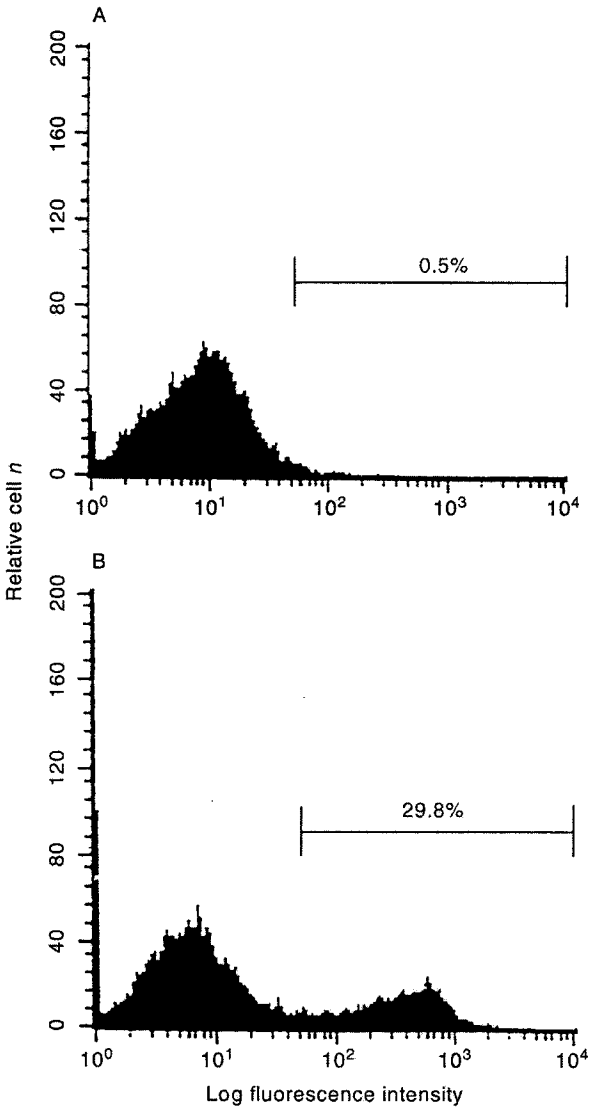


**Fig. 3.** Annexin V labeling of CJ7 embryonic stem (ES) cells. Apoptosis was assessed upon interferon (IFN) treatment of ES cells where (A) was the medium and (B) was IFN-γ. After 24 h in the absence (A) or in the presence (B) of 500 U/mL IFN-γ, ES cells were labeled with fluorescein isothiocyanate (FITC)-labeled Annexin V. Values represent the percentage of Annexin V-positive cells.

### *IFN-γ affected ES cell growth through apoptosis*

In order to determine whether IFN-γ induced ES cell death by apoptosis, cells were labeled with Annexin V. Indeed, the cell staining with Annexin V can be used as a specific probe for apoptosis in the early phase when the cell membrane is still intact (Koopman *et al.* 1994; Darzynkiewicz *et al.* 1997). As assessed by flow cytometry analysis, after culture with 500 U/mL of IFN-γ for 24 h, ES cells were about 30% Annexin V positive as compared with 0.5% Annexin V positive ES cells from untreated culture (Fig. 3A,B). Also, as shown in Fig. 4, gel electrophoresis of DNA extracted from IFN-treated cell culture revealed characteristic internucleosomal DNA cleavage for fragments that were multiples of 180~200 bp (lane 2, 500 U/mL of IFN-γ; lane 3, 1000 U/mL of IFN-γ). Under optimal

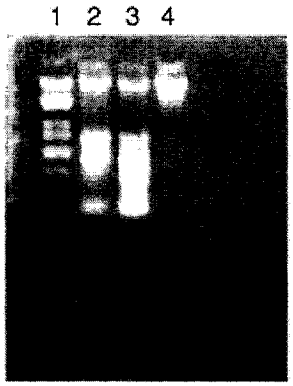


**Fig. 4.** Gel analysis of DNA extracted from CJ7 embryonic stem (ES) cells. The DNA was extracted from untreated ES cells after culture for 24 h without (lane 4), with 500 U/mL of interferon (IFN)-γ (lane 2) or with 1000 U/mL of IFN-γ (lane 3) and submitted to gel electrophoresis on a 1% agarose gel. A molecular weight marker is displayed on lane 1, DNA fragments were visualized with ethidium bromide staining.

growth conditions (15% FCS/DMEM) no spontaneous DNA fragmentation occurred (lane 4).

### *ES cells expressed Bcl-2*

Flow cytometry was used to examine Bcl-2 expression in ES cells exposed or not to IFN-γ, showing expression of Bcl-2 as a unimodel peak of staining (Fig. 5B). The mean fluoresence intensity of Bcl-2-labeled ES cells was significantly higher when compared to control IgG-labeled ES cells (average mean fluorescence intensity (mFI) for IgG and anti-Bcl-2 mAb were $4.54 \pm 0.3$ and $6.48 \pm 0.5$, respectively, $n = 4$; $P < 0.01$).

### *Bcl-2 protein and Fas antigen level in ES cells exposed to IFN-γ*

To investigate Bcl-2 involvement in ES cell apoptosis triggered by IFN-γ, we tested its expression by flow cytometry. As depicted in Fig. 5A, two cell populations were gated based on FSC versus SSC analysis: The R1 gate corresponds to viable ES cells and the R2 gate corresponds to cells undergoing a cell death process. From these figures, we first observed the appearance of the R2-gated cell population upon treatment with IFN-γ. As compared to both R1-gated untreated cells (Fig. 5B) and R1-gated IFN-treated cells (Fig. 5A), Bcl-2

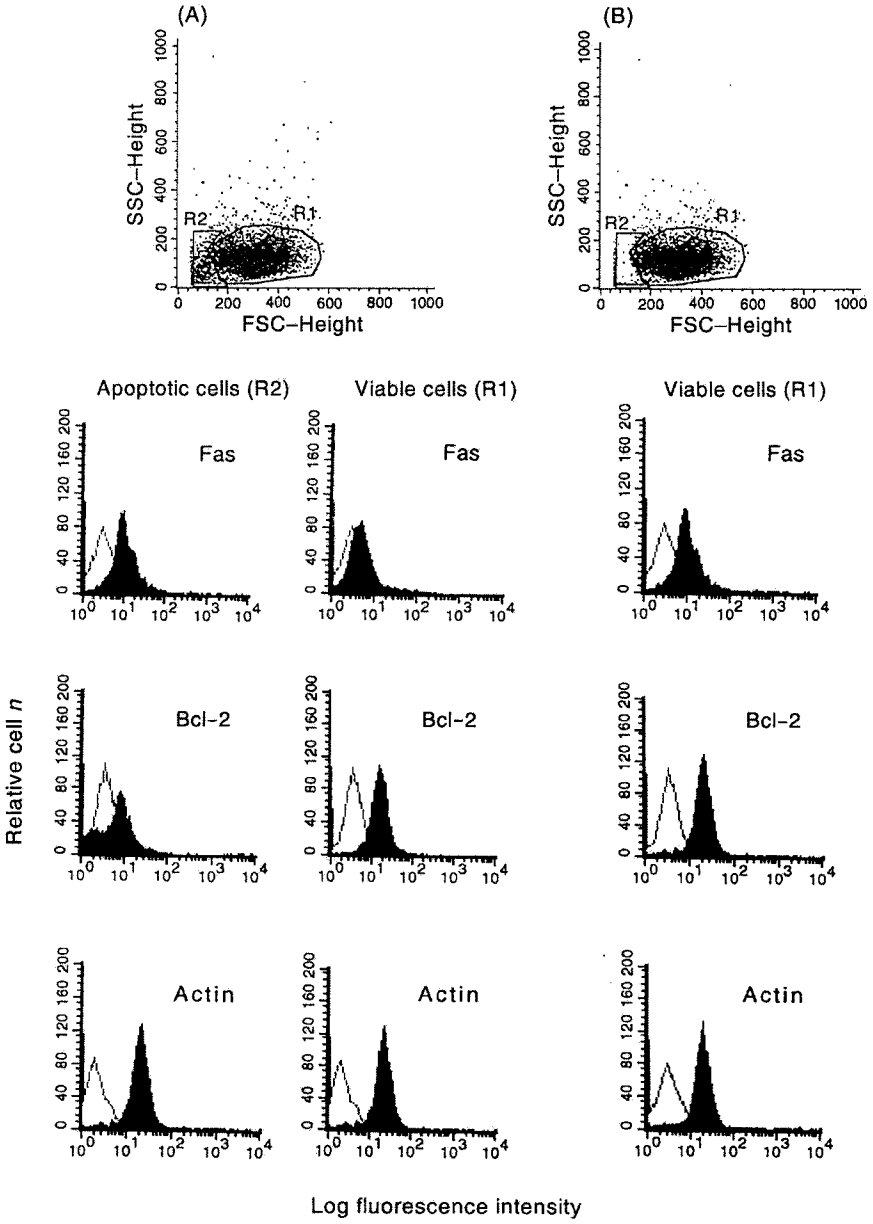


**Fig. 5.** Expression of Bcl-2 protein in cultured embryonic stem (ES) cells. The CJ7 ES cells were treated (A) or not (B) with interferon (IFN)-γ at 500 U/mL for 24 h and were either labeled with anti-Fas monoclonal antibody (mAb) or anti-actin Abs, or permeabilized prior to being labeled with anti-Bcl-2 mAb, as described in the Materials and Methods. The result of one representative experiment out of four is illustrated. (□), ES cells labeled with an irrelevant immunoglobulin G (IgG) Ab, as negative control. (■), ES cells labeled with anti-Fas, Bcl-2 or actin Abs, as noted. In the upper dot plots, the R1 gate delineates viable cells while R2 delineates cells undergoing a death process.

**Table 1.** Percentage of Bcl-2-positive cells upon interferon (IFN)-γ treatment

| | R1 (viable cells) | R2 (apoptotic cells) |
|---|---|---|
| Medium | 37 ± 2.6 | No apoptotic cell population** |
| IFN-γ 500 U | 36.7 ± 1.1* | 19.7 ± 2.1† |
| IFN-γ 1000 U | 35.3 ± 2.02* | 12.7 ± 2.1‡ |

Values represent the mean ± SD of three independent experiments.

*Not significant when compared with control (medium) groups.

†$P < 0.001$.

‡$P < 0.01$ when compared with viable cells.

**Without IFN-γ, most of the cells are distributed in the R1 region and no cells were distributed in the R2 region.

as well as Fas expression were down-modulated in R2-gated IFN-treated cells (Fig. 5A). By contrast, actin staining was not modified in either cell population. Table 1 summarizes the results obtained from three independent experiments performed in CJ7 ES cells. The percentage of Bcl-2-positive cells in cultured ES cells treated by IFN-γ was significantly higher in viable cells (R1) than in the apoptotic cell population (R2). In addition, no variation was observed between untreated and IFN-treated R1 cells. Actin levels were similar between viable cells and the apoptotic cell population after IFN-γ treatment.

## Discussion

A large body of data suggests that embryonic cells may undergo apoptosis. Kircheis *et al.* (1996) reported that early embryonic cells and early mouse embryos were selectively lyzed by the alternative complement pathway, which indicated a possible mechanism of programmed cell death in embryogenesis. Pardo *et al.* (1996) found that transfection of cyclin D1, a G1/S cyclin, into rat embryo cells (REC) results in a cellular population that overexpresses cyclin D1, is morphologically transformed, and demonstrates an increased incidence of apoptosis. During *in vivo* rat embryo development, a zinc deficiency causes apoptosis in embryo cells and results in embryo cell death (Rogers *et al.* 1995). The IFN-γ is a toxic factor during early embryo development. Early mouse embryo development is inhibited by IFN-γ under *in vitro* culture conditions (Hill *et al.* 1987); however, the further mechanism of IFN-γ embryotoxicity has not been demonstrated completely. In the present study, we found that IFN-γ could inhibit ES cell proliferation and this inhibition resulted in apoptosis, which was confirmed by Annexin V flow cytometry analysis and DNA ladder electrophoresis (Figs 3,4).

The triggering of Fas molecules with anti-Fas mAb is known to induce apoptosis in Fas-expressing cells (Matsumoto *et al.* 1995; Rokhlin *et al.* 1997). In a recent study, we found that ES cells expressed Fas antigen but not Fas ligand, and anti-Fas monoclonal antibody could induce ES cell apoptosis (Zou *et al.* 2000, in press). By western blot analysis, Xu *et al.* (1998) found that Fas expression was dramatically enhanced in HT29 cell lines after the administration of IFN-γ at a concentration of 100 U/mL; however, Fas expression was slightly decreased when IFN-γ was used at high concentration (> 500~1000 U/mL), probably because of the increased cell death. Using flow cytometry analysis, we failed to find that IFN-γ upregulates either Fas expression or FasL in ES cells when the dose of IFN-γ was 50~100 U/mL (data not shown). In addition, in the cytotoxicity study, we did not find that anti-Fas mAb (Jo2) had synergistic effects in inducing ES cell death. However, the present data clearly showed a cooperative effect in inducing ES cell death (Fig. 2).

Proteins of the Bcl-2 family are intracellular membrane-associated proteins that regulate programmed cell death (apoptosis) either positively or negatively (Kroemer 1997). Flow cytometry is an effective method to detect Bcl-2 expression in mouse cells (Veis *et al.* 1993; Matsumoto *et al.* 1997; Zimowska *et al.* 1997). The current study presents, we believe, the first analysis of Bcl-2 expression in mouse embryonic stem cells by flow cytometry (Fig. 5). The analysis showed that 20~45% of undifferentiated ES cells were labeled with Bcl-2 mAb. Interestingly, Saponin was effective in the permeabilization for Bcl-2 staining in the present study. In contrast, 0.1% Triton was ineffective for permeabilization, impairing Bcl-2 detection by flow cytometry under this condition (data not shown). The function of Bcl-2 in mouse embryo development remains undefined. The fact that mouse ES cells express Bcl-2 indicates that Bcl-2 may be involved in early embryo differentiation and organogenesis by regulating apoptosis and clonal cell selection. Novack & Korsmeyer (1994) found that Bcl-2 was widely distributed in the developing mouse and suggested that Bcl-2 may have roles beyond regulation of developmental cell death.

Piacentini *et al.* (1994) demonstrated Bcl-2 changes in rat uterine cells during embryo implantation. In another report, the cyclic expression of Bcl-2 in human uterine endometrium was found during the menstrual cycle. In chicken spermatogenesis, Bcl-2 is highly expressed in immature testis enriched in spermatogonia, but barely detectable in mature testis (Vilagrasa *et al.* 1997). In the mouse embryo, neither bax nor bcl-x mRNA was expressed in the sensory epithelium from embryonic day (E) 13–19. In contrast to this pattern, Bcl-2 mRNA was expressed by E15–E19 as

shown by *in situ* hybridization (Ishii *et al.* 1996). Flow cytometry is able to detect changes in Bcl-2 levels after apoptosis has begun by using forward and side scatter to separate apoptotic cell and viable cell populations (Tamaru *et al.* 1993; Pepper *et al.* 1998). We report here that after incubation with IFN-γ, the level of Bcl-2 expression in apoptotic ES cells is decreased, as detected by flow cytometric analysis. However, in the surviving population of ES cells, the level of Bcl-2 expression was not changed compared to untreated ES cells. We did not find increased Bcl-2 levels in the surviving ES cells, which had been demonstrated in other reports (Zimowska *et al.* 1997). One could postulate that ES cells are heterogeneous in Bcl-2 level, explaining the different response of ES cells to IFN-γ treatment. To rule out the possibility that low Bcl-2 expression could be directly related to the impairment of total protein synthesis during the apoptosis process, we stained our cells with anti-actin Abs. As shown in Fig. 5, both surviving and apoptotic IFN-treated cells displayed a rather similar level of actin expression.

In conclusion, the present study demonstrates that: (i) IFN-γ induces apoptosis in ES cells; (ii) mouse ES cells express Bcl-2; and (iii) apoptotic cells expressed lower level of Bcl-2 compared with the surviving ES cell population after IFN-γ treatment. This could partly explain the mechanism of IFN-γ embryotoxicity and further studies are worthwhile to determine its *in vivo* role in embryo development.

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